Reflectance Spectrophotometry of Pyridine Hemochromes in Frankfurters

Jay B. Fox, Jr.,* Michael Dymicky, Susan Keyser, and Aaron E. Wasserman

Varying the kind and position of ring substituents on pyridine when substituted for nitrite in frankfurter emulsions produced colors in frankfurters that ranged from orange through red to purple, with different degrees of stability. Reflectance spectroscopy, using the proper reference standards, showed the presence of oxymyoglobin and oxyhemoglobin absorption bands in frankfurters made with weakly complexing pyridine derivatives, suggesting protein stabilization by the derivative. Reflectance absorption bands in the 660–700-nm region in greenish rings immediately beneath the surface of finished frankfurters are evidence of the pyridine-catalyzed formation of verdohemochromes. Color formation in frankfurters cannot always be predicted from model systems. Color development in frankfurters varied from batch to batch and, although dependent on, was not guaranteed by the presence of reductants.

The pink color of cured meat products is developed by the reaction of nitric oxide, derived from nitrite, with the heme pigments of the meat. Although a number of compounds have been proposed as nitrite color substitutes in cured meats (Tarladgis, 1967; Howard *et al.*, 1973), these compounds have not been tested comprehensively in meat products. A preliminary survey in this laboratory of a number of reactive compounds, including nitrogenous heterocyclic compounds, indicated that pyridine derivatives were in general the most effective. Pyridine is the simplest heterocyclic nitrogenous base with aromatic character, is comparatively readily substituted, and many derivatives are commercially available. Pyridine and those of its derivatives that have been studied toxicologically, while mildly irritating and/or obnoxious, are not acutely toxic, even at high doses. The major toxic effect of pyridine derivatives is their action as niacin antimetabolites but this effect, even as the generally offensive odors and flavors, might be eliminated by chemical modification once the ideal color-forming characteristics have been established. We therefore selected substituted pyridine compounds for systematic tests of the effects of the kind and position of various substituents on frankfurter color. This paper reports the results of the visual and spectrophotometric evaluation of the color of frankfurters prepared with pyridine derivatives.

EXPERIMENTAL SECTION

Frankfurter emulsions, containing 11% protein and 30% fat, were prepared with all cure ingredients except nitrite. The pyridine derivatives tested and reductants were dissolved in a minimal amount of ethanol, water, or mixtures of the two, and worked into weighed portions of emulsion. The emulsion was stuffed in cellulose frankfurter casing, linked, and processed according to the schedule of heating and smoking suggested by Drying Systems Co. The frankfurters were processed to a final internal temperature of 160°F (71.1°C).

The color of the frankfurters was assessed visually immediately after processing and several times during storage at 4°. Reflectance spectra were recorded with a Cary 14 spectrophotometer equipped with a microspecular accessory. With a device which collects light at a specific angle, such as the microspecular accessory, the scattering of light in diffuse reflectance is critical since a change in the scatter envelope with wavelength will change the apparent light absorption. Initially, the reflectance of a freshly cut surface of MgO was used as a reference, setting the R_A values (reflected light measured on an absorbance scale) to zero on the absorbance scale by adjusting the multipots of the Cary 14. Snyder and Armstrong (1967) showed that the amount of light reflected from a 5% solution of nonfat dry milk compared to that reflected from a MgO surface decreases with increasing wavelength between 400 and 700 nm (increasing R_A). The value of R_A similarly increased when the reflectance spectra of the cut surface of frankfurters were compared to the spectrum of a MgO reference. Therefore, a reference was needed that would have scatter characteristics closely resembling those of frankfurters. Bleaching the cut surface of a frankfurter with 30% H_2O_2 produced a reference standard which eliminated the skew in the sample curves. Fresh reference standards were prepared for each run. Spectra of frankfurters without nitrite were similar to those of Tappel (1957) and Tarladgis (1962a) for the denatured heme pigments of fresh meat. Frankfurters with nitrite had the same reflectance spectra as the spectrum reported by Tarladgis (1962b) for the nitric oxide hemochrome of ham.

For transmission spectra of the pyridine ferrohemochromes, we prepared the sodium lauryl sulfate denatured globin hemochromes as suggested by Howard *et al.* (1973). The 0.025 m*M* metmyoglobin was completely denatured and solubilized with 0.1% sodium lauryl sulfate (SLS) at pH 5.5 (Fox *et al.*, 1975). We prepared pigment solutions containing 25 m*M* of the pyridine derivative. Spectra of the solutions were recorded in a Cary 14 spectrophotometer.

RESULTS

Visual Observations. Table I summarizes the data on formation and stability of color in frankfurters prepared with the pyridyl derivatives tested. Addition of pyridine to the emulsion produced a pink color which faded rapidly. Substituents on the pyridine ring affected both color and stability. No pink color developed with any of the 2-substituted pyridines, probably due to steric hindrance. No color change occurred with the aminopyridines, and 3-hydroxypyridine produced a greenish-tan color. All other substituted pyridines produced a frankfurter that was some shade of red. 4-Methylpyridine produced a bright orange frankfurter; acetyl, formyl, and carboxymethyl groups in the 4 position and 3-acetylpyridine produced purple frankfurters. All others gave a pink color.

The color produced was not uniform. Just beneath the surface, all frankfurters had a greenish-tan ring which was narrowest in the purple frankfurters and broadest in the pink frankfurters. With added ascorbate, the width of the ring was reduced to 1 mm or less in the purple frankfurters. In the pink frankfurters, ascorbate reduced the width of the discolored ring, from about ⅔ of the diameter of the

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118.

Table I. Pyridine Derivatives and Frankfurter Color

Compound	Color	Stability in air
Control (no color agent)	Tan	
$NaNO_2$	Pink	Stable
Pyridine	Pink	1–5 min
2-Methylpyridine	Tan	
3-Methylpyridine	Pink-tan	0.5–1 hr
4-Methylpyridine	Orange	0.5–1 hr
2-Formylpyridine	Tan	
3-Formylpyridine	Pink	1–5 min
4-Formylpyridine	Purple-pink	1–5 min
2-Carboxymethylpyridine	Tan	
3-Carboxymethylpyridine	Pink	1–5 min
4-Carboxymethylpyridine	Pink (purple)	1–5 min
2-Acetylpyridine	Tan	
3-Acetylpyridine	Purple	1–2 hr
4-Acetylpyridine	Purple	1–2 hr
2-Hydroxypyridine	Tan	
3-Hydroxypyridine	Green-tan	
4-Hydroxypyridine	Tan-pink	1–5 min
2-Aminopyridine	Tan	
3-Aminopyridine	Tan	
4-Aminopyridine	Tan	
Isoquinoline	Purple	1–2 hr

frankfurter with 0.05% ascorbate to 1 mm or less with 0.5 or 5.0% ascorbate. Discoloration was not strictly related to the color of the pyridine ferrohemochrome; isoquinoline produced a purple color, but the discolored ring was 2–3 mm wide and had a strong greenish cast.

The size and intensity of the color of the pink core decreased with storage. Frankfurters were stored at 4° for periods up to 30 days in glass jars or wrapped in Saran. In 2 weeks neither the nitrite-cured nor the purple pyridine-containing frankfurters faded appreciably. In the rest, the pink core decreased to less than half its original size. Furthermore, whether fresh or stored, the pink frankfurters faded rapidly upon exposure to air, turning brown in a few minutes.

Surface color was variably affected by the pyridine derivatives. In general, the surface was yellowish-brown as compared to the reddish-brown of the nitrite-cured frankfurters. Added reductants caused fading of the surface colors. Increasing cysteine concentrations from 0.05 to 5.0% resulted in a gradation from yellowish-brown to white. Ascorbate (5.0%) produced a pale brown surface.

Two other anomalies were observed. In one batch of frankfurter emulsion, the addition of substituted pyridines did not produce any color, although the nitrite containing sample developed the usual pink hue. Addition of reductants up to 5% did not develop the color. Furthermore, the color of the SLS-denatured globin ferrohemochrome was not necessarily duplicated in the hue of the frankfurter. Table II lists the compounds that did not reproduce the ferrohemochrome color in the frankfurters.

Spectrophotometric Analysis. The reflectance spectra of the red-hued frankfurters showed the typical pyridine ferrohemochrome bands at 525–530 and 555–560 nm. Principal variations included differences in the intensity of absorption in the regions 450–500 and 600–700 nm, the occasional appearance of a band at 580 nm, and variations in the overall intensity of the absorption. The spectral differences between the pink frankfurter with 3-carboxymethylpyridine and the purple frankfurter with 3-acetylpyridine are shown in Figure 1. Normally, the pink were lighter than the purple, their reflectance spectra being 0.01–0.02 absorbance unit less overall. For purposes of illustration, the curves were normalized to each other at 600 nm, giving approximately equal intensity of light absorption in the red region. Relative to the purple pigment (Figure 1), the pink pigment absorbs more light in the 450–500-nm region or, conversely, reflects less blue light. This increased reflectance in the blue region of the purple accounted for the hue. Figure 1 also shows the reflectance spectrum of a frankfurter made with 4-methylpyridine, and the increased absorption in the 450–500-nm region that produced the orange hue of the frankfurter.

Table II. Color Differences between SLS Hemochrome and Frankfurter Color of Several Pyridine Derivatives

Pyridine substituent	Color	
	SLS hemochrome	Frankfurter
3-Methyl	Orange	Pink-tan
3-Acetyl	Rose	Purple
3-Hydroxy	Red-orange	Green-tan
3,4-Amino	Red	Tan

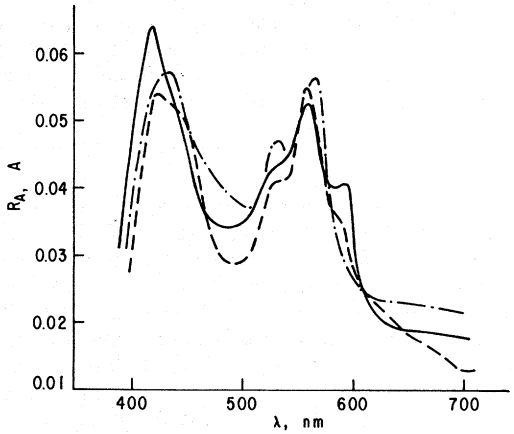

Figure 1. Comparison of R_A spectra of typical purple and pink frankfurters produced with pyridine derivatives: (—) 3-carboxymethylpyridine (pink); (-·-) 4-methylpyridine (orange); (– – –) 3-acetylpyridine (purple).

The variations in the reflectance spectra were due to the presence of absorption bands on either side of the principal pyridine ferrohemochrome bands, as seen in the transmission spectra of the SLS-denatured globin hemochromes, Figure 2 (Fox *et al.*, 1975). Since the same spectral features were common to all ferrohemochromes of a given hue, the pyridine derivatives used for the spectra of Figure 2 were not necessarily the same as those used for the spectra of Figure 1. The pigment concentration is the same for the three spectra, so the true spectral relations are seen, but the same relative absorption values in the blue and red regions are observed for the three different hues. These bands are due to charge transfer between the iron and the ligand (Brill and Williams, 1961) and their position and significance have been discussed by Fox *et al.* (1975).

The main difference between the reflectance spectra of the frankfurters made with a given substituted pyridine (Figures 1 and 3) and the transmission spectra of the corresponding SLS-denatured globin hemochrome (Figure 2) was an absorption band at 580 nm in the former. This band is due to the presence of oxymyoglobin in the frankfurters, and means that the pigment was not fully denatured in the cooking process since the denatured protein pigment cannot form the oxygenated compound. Bands at 580 nm never appeared in the control frankfurters (no nitrite) implying that the pigment was fully denatured. We concluded from these observations that the pyridine de-

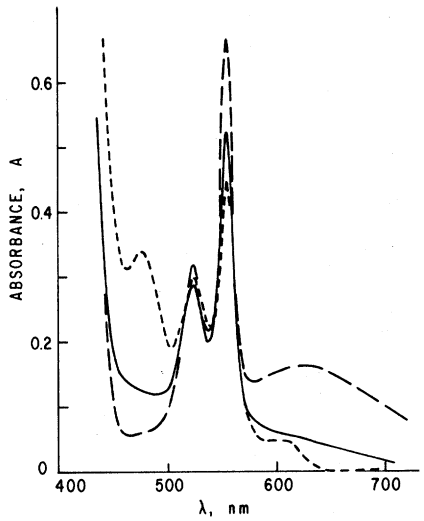

Figure 2. Transmission spectra of substituted pyridine SLS-denatured globin hemochromes: (—) 3-acetylpyridine (rose); (– – –) 4-carboxymethylpyridine (purple); (- - -) 3-methylpyridine (orange).

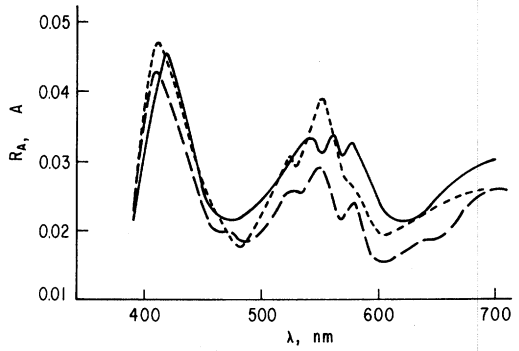

Figure 3. Comparison of R_A spectra of frankfurters containing (—) 2-formylpyridine; (- - -) 3-carboxymethylpyridine, surface freshly cut; (– – –) same faded after exposure to air.

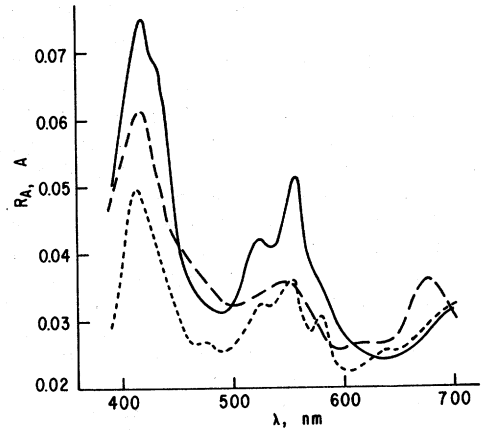

Figure 4. R_A spectra of core and ring of the cut surface of a frankfurter containing 3-acetylpyridine: (—) core; (– – –) ring; (- - -) faded 3-carboxymethylpyridine hemochrome.

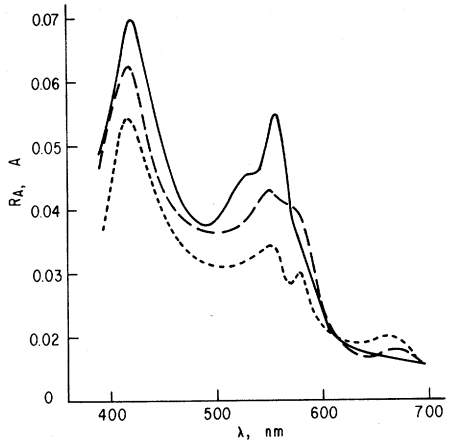

Figure 5. Spectra of frankfurters containing 3-formylpyridine and (- - -) 0.05%, (– – –) 0.5%, and (—) 5.0% ascorbate. Stored 3 weeks at 4°.

rivatives protected the globin against denaturation. This was true even for those pyridine derivatives that did not form, or formed only weak hemochromes. The solid curve of Figure 3 is the reflectance spectrum of a frankfurter made with 2-formylpyridine and shows peaks at 545 and 580 nm in addition to a weak pyridine hemochrome peak at 560 nm. The figure also shows the conversion of the pink hemochrome of 3-carboxymethylpyridine (dotted curve) to oxymyoglobin (dashed curve) on exposure to air. The figure also shows that the middle absorption band maxima has a different wavelength of maximum absorbance (λ_{max}) for the two different substituted pyridine hemochromes. Variations in λ_{max} were first reported by Howard *et al.* (1973) and are related to the effect of the substituent on the pyridine ring on the bonding and antibonding orbitals of the π-electron system of the pyridine ring (Fox *et al.*, 1975).

Discoloration. Fading and heme oxidation were the principal identifiable causes of discoloration of the pyridine-containing frankfurters. Figure 4 shows the spectra of the pink core of a frankfurter prepared with 3-acetylpyridine and the greenish ring just beneath the surface. The spectrum of a faded frankfurter prepared with 3-carboxymethylpyridine is shown for comparison. The spectrum of the discolored ring lacks a pyridine hemochrome maxima in the 555–560-nm region and has the characteristic absorption band at 675–680 nm which is typical of the pyridine-catalyzed oxidized hemochrome (Lemberg and Legge, 1949). Heme oxidation catalysis varied from base to base; 3-hydroxypyridine discolored the whole pigment in the frankfurters while isoquinoline produced a deep green ring. Spectral evidence did not indicate that the rapid discoloration upon exposure to light resulted in heme oxidation, but after storage for 3 weeks at 4° a slow increase in the absorption in the 660–680-nm region indicated that some heme oxidation was taking place. Increasing concentration of reductant reduced the amount of discoloration (Figure 5). At the lowest ascorbate concentration the spectrum shows that the red pigment was not the pyridine hemochrome, but was oxymyoglobin.

DISCUSSION AND EVALUATION

Results from this study indicate that the pigment produced in frankfurter emulsions treated with pyridine derivatives is the denatured-globin, substituted-pyridine ferrohemochrome. This conclusion agrees with that of Howard *et al.* (1973). Our results, however, indicate that the variations in hue are due to the presence of specific absorption bands rather than to shifts in the major absorption of the pyridine hemochrome as suggested by Howard *et al.* (1973). We have also found that under conditions of practice, more is involved than formation of the hemochrome. The occasional failure of color to form and variations in hue between the SLS-denatured globin, substituted-pyridine hemochrome produced under ideal conditions, and the pigment produced in frankfurters from the same pyridine derivative under less controlled conditions, illustrate the dangers in extrapolating from a model system to the product. The reason for these variations is not

fully understood, but Akoyunoglou *et al.* (1963) had difficulty determining the equilibrium constants of the nitrogenous base hemochromes they studied, and Fox *et al.* (1975) could not determine the rate constants for formation of substituted-pyridine, SLS-denatured globin hemochromes. Since the pigments are hemochromes (ferrous), reductants are necessary, but the addition of reductants will not necessarily improve color, as Howard *et al.* (1973) also observed. Reductants do, however, increase stability of the pyridine hemochrome. The role of reductants in heme pigment chemistry is ambiguous for there are a number of green heme pigments that are specifically produced by oxidation in the presence of reductants, choleglobin with ascorbate and sulfmyoglobin and sulfhemoglobin with cysteine (Lemberg and Legge, 1949).

Reflectance spectrophotometry is a useful tool in corroborating visual observations and identifying specific pigments. The comparatively small area which the microspecular attachment examines has been particularly useful in determining the spectra of pigments that occur only over a limited area, such as the discolored rings observed in the frankfurters of this study. Although the reflected intensities of light from this device are low, the spectra are sufficiently distinctive to allow positive identification of specific pigments.

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